

**Piperacillin/tazobactam resistance in a clinical isolate of *Escherichia coli* due to IS26-mediated amplification of *bla<sub>TEM-1B</sub>*.**

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## **Supplementary Methods 1:**

### *DNA extraction*

Genomic DNA for qPCR was extracted using the PureGene® Yeast/Bact kit B (Qiagen, Germany) following the manufacturer's instructions for extraction of DNA from Gram-negative bacteria after normalising each culture to an optical density at 600nm ( $OD_{600}$ ) of 1 and eluted in molecular grade water.

Long fragment genomic DNA extraction for PCR amplification and both Illumina and Oxford Nanopore Technologies (ONT) sequencing was performed using the Genomic DNA Buffer Set, following the manufacturer's instructions for genomic DNA extraction using the Genomic-tip 100/G (both Qiagen, Germany) but eluted in molecular grade water.

Plasmid extraction was performed from 100 ml LB broth (LB) cultures with the Plasmid Midi Kit using Genomic-Tip 100 (both Qiagen, Germany) following the manufacturer's instructions and eluted in molecular grade water.

Total DNA concentration was measured using a QuBit 4.0 Fluorometer (Invitrogen, US), with the dsDNA Broad Range kit.

### *PCR*

A single replicate of all PCR reactions was performed using 0.02 U/ $\mu$ l Q5® High-Fidelity DNA Polymerase, 1x Q5® Reaction Buffer, 200  $\mu$ M dNTPs and 0.5  $\mu$ M of each primer (Supplementary Table 4) in a total volume of 50  $\mu$ l.

16S rRNA PCR amplification was performed using the following protocol: denaturation at 98°C for 30 seconds, followed by 45 cycles of denaturation at 98°C for 10 seconds, annealing at 53°C for 30 seconds and elongation at 72°C for 30 seconds, followed by a single, final extension of 2 minutes at 72°C.

The detection of the pseudo-compound transposon or translocatable unit (TU) was performed using the following PCR protocol: denaturation at 98°C for 30 seconds, followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 68°C (left junction of the pseudo-compound transposon in the chromosome and the tandem array of TUs) or 67°C (right junction of the pseudo-compound transposon in the chromosome) for 30 seconds and elongation at 72°C for 17 seconds (left junction of the pseudo-compound transposon in the chromosome), 25 seconds (right junction of the pseudo-compound transposon in the chromosome) or 20 seconds (tandem array of TUs), followed by a single, final extension of 2 minutes at 72°C.

Detection of the formation of junctions in the pHSG396:IS26 due to the insertion of the TU using the following PCR protocol: denaturation at 98°C for 30 seconds, followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 68°C (left junction of the insertion) or 67°C (right junction of the insertion) for 30 seconds and elongation at 72°C for 15 seconds (left junction) and 14 seconds (right junction), followed by a single, final extension of 2 minutes at 72°C.

The PCR products from the left side of the pseudo-compound transposon in the chromosome and the TU were PCR purified using the Monarch® PCR and DNA Clean-up Kit (New England Biolabs (NEB), USA) following the manufacturer's protocol and eluted in molecular grade water. The PCR products from the right side of the pseudo-compound transposon in the chromosome and the left and right junction of the TU insertion into pHSG396:IS26 were extracted from the gel using the Monarch® DNA Gel Extraction Kit (NEB, USA) following the manufacturer's instructions and eluted in molecular grade water. All purified DNA extracts were then Sanger sequenced by GeneWiz (Takley, UK).

#### *IS26 plasmid construct*

IS26 from 190963 (TZP-susceptible isolate) was amplified using 0.02 U/μl Q5® High-Fidelity DNA Polymerase, 1x Q5® Reaction Buffer, 200 μM dNTPs and 0.5 μM of the primers CMCap\_F and CMCap\_R (Supplementary Table 4) in a total volume of 50 μl. Amplification was performed using the

following protocol; Denaturation at 98°C for 30 seconds, followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 69°C for 30 seconds and elongation at 72°C for 9 seconds, followed by a single, final extension step 72°C for 2 minutes.

The restriction sites for the restriction enzymes EcoRI and KpnI were added to the 5' ends of the 820 bp double stranded amplicon by a second round of PCR, using the primers CMCapRE\_F and CMCapRE\_R (Supplementary Table 4). Amplification was performed using the same protocol as the initial amplification of IS26, except with an annealing temperature of 72°C for 30 seconds.

The resulting PCR product and the recipient plasmid pHSG396 were digested with the restriction enzymes EcoRI and KpnI (both NEB, US). PCR clean-up was then performed using the Monarch PCR and DNA Clean-up Kit following the manufacturer's instructions. The digested IS26 PCR product and pHSG396 were ligated together using T4 ligase (NEB, US), at a 3:1 insert to vector mass ratio, incubated at room temperature for 10 minutes.

The ligated plasmid (5 µl) was transformed into NEB® 5-alpha competent *E. coli* (NEB, US) using the following protocol; the reaction was incubated on ice for 2 minutes, then heat shocked at 42°C for 30 seconds. Following a second incubation on ice for 2 minutes, 950 µL of SOC outgrowth medium was added and incubated at 37°C, 250 rpm for 1 hour. The transformed cells were plated out onto LB agar supplement with 35 µg/ml chloramphenicol and Isopropyl β-D-1-thiogalactopyranoside/X-gal (Fisher Scientific, USA) and incubated at 37°C for 18 hours and the plasmid was extracted following the protocol above. IS26 in pHSG396 was Sanger sequenced by GeneWiz (Takely, UK) using commercially available M13 primers.

**Supplementary Table 1: Resistance profile of paired isolates determined in clinic.** Resistance profile of cefpodoxime (CPD), cefoxitin (FOX), piperacillin/tazobactam (TZP), meropenem (MEM), ciprofloxacin (CIP), cefotetan (CTT), amikacin (AMK), ertapenem (ETP), amoxicillin/clavulanic acid (AMC), chloramphenicol (CHL) and ampicillin (AMP) towards identified paired isolates with presumptive clonality assessed in clinic by disk diffusion method. Disk diffusion antimicrobial susceptibility testing was performed as a single replicate ( $n=1$ ).

Patient	Isolate	CPD	FOX	TZP	MEM	CIP	CTT	AMK	ETP	AMC	CHL	AMP
Number												
1	101737	S	S	S	S	S	S	S	S	S	-	-
	102167	S	S	R	S	R	S	S	S	I	-	-
2	153964	S	S	S	S	S	S	S	S	R	S	R
	152025	S	S	R	S	S	S	S	S	R	S	R
3	190693	S	S	S	S	R	R	S	S	R	S	R
	169757	S	S	R	S	R	R	S	S	R	S	R

**Supplementary Table 2: TEM-1B is likely to be the most important determinant of piperacillin/tazobactam resistance.** Minimum inhibitory concentrations (MIC) of piperacillin supplemented with 4 µg/ml tazobactam, 8 µg/ml tazobactam, 16 µg/ml tazobactam and 4 µg/ml tazobactam plus 100 mM sodium chloride. Each MIC were performed in triplicate ( $n=3$ ).

	Piperacillin plus 4 µg/ml tazobactam	Piperacillin plus 8 µg/ml tazobactam	Piperacillin plus 16 µg/ml tazobactam	Piperacillin plus 4 and 100 mM NaCl
TZP-resistant	128 µg/ml	64 µg/ml	16 µg/ml	64 µg/ml

**Supplementary Table 3: Mean and standard error of the mean of all data presented in this study.**

Assay and figure	Isolate	Gene	Media	Mean	Standard Error of the mean ( $\pm$ )
<b>Nitrocefin assay</b>					
Fig. 1A	TZP-susceptible	-	-	0.041	0.002
Fig. 1A	TZP-resistant	-	-	0.208	0.011
<b>qPCR</b>					
Fig. 1B	TZP-susceptible	aac(3)-lla	-	1.089	0.037
		aac(6')-lb-cr	-	1.226	0.068
		blaOXA-1	-	1.516	0.02
		blaTEM-1B	-	1.207	0.047
		tet(D)	-	0.525	0.016
Fig. 1B	TZP-resistant	aac(3)-lla	-	8.523	0.105
		aac(6')-lb-cr	-	8.2	0.139
		blaOXA-1	-	7.252	0.375
		blaTEM-1B	-	9.484	0.354
		tet(D)	-	9.154	0.286

Fig. 3	TZP-susceptible - no TZP	aac(3)-lla	-	0.734	0.062
		aac(6')-lb-cr	-	0.875	0.005
		blaOXA-1	-	1.199	0.041
		blaTEM-1B	-	1.002	0.063
		tet(D)	-	1.292	0.104
Fig. 3	TZP-susceptible - TZP	aac(3)-lla	-	3.104	0.339
		aac(6')-lb-cr	-	3.249	0.377
		blaOXA-1	-	3.708	0.685
		blaTEM-1B	-	3.604	0.811
		tet(D)	-	5.641	0.785
Fig. 3	TZP-susceptible + IS26 - TZP	aac(3)-lla	-	6.37	0.468
		aac(6')-lb-cr	-	7.379	0.355
		blaOXA-1	-	8.571	0.369
		blaTEM-1B	-	10.068	0.896
		tet(D)	-	10.919	1.153

Fig. 3	TZP-susceptible + IS26 - CHL only	aac(3)-Ila	-	-0.627	0.297
		aac(6')-lb-cr	-	1.128	0.186
		blaOXA-1	-	0.8	0.283
		blaTEM-1B	-	1.257	0.105
		tet(D)	-	0.087	1.284
Comparative fitness					
Fig. 4	TZP-susceptible	-	ISO	0.891	0.051
	TZP evolved	-	LB	1.105	0.062
		-	M9	1.068	0.033
Fig. 4	TZP-resistant	-	ISO	0.894	0.055
		-	LB	1.051	0.031
		-	M9	0.933	0.14

**Supplementary Table 4: Primer name and sequences used for PCR amplification and qPCR in this study.**

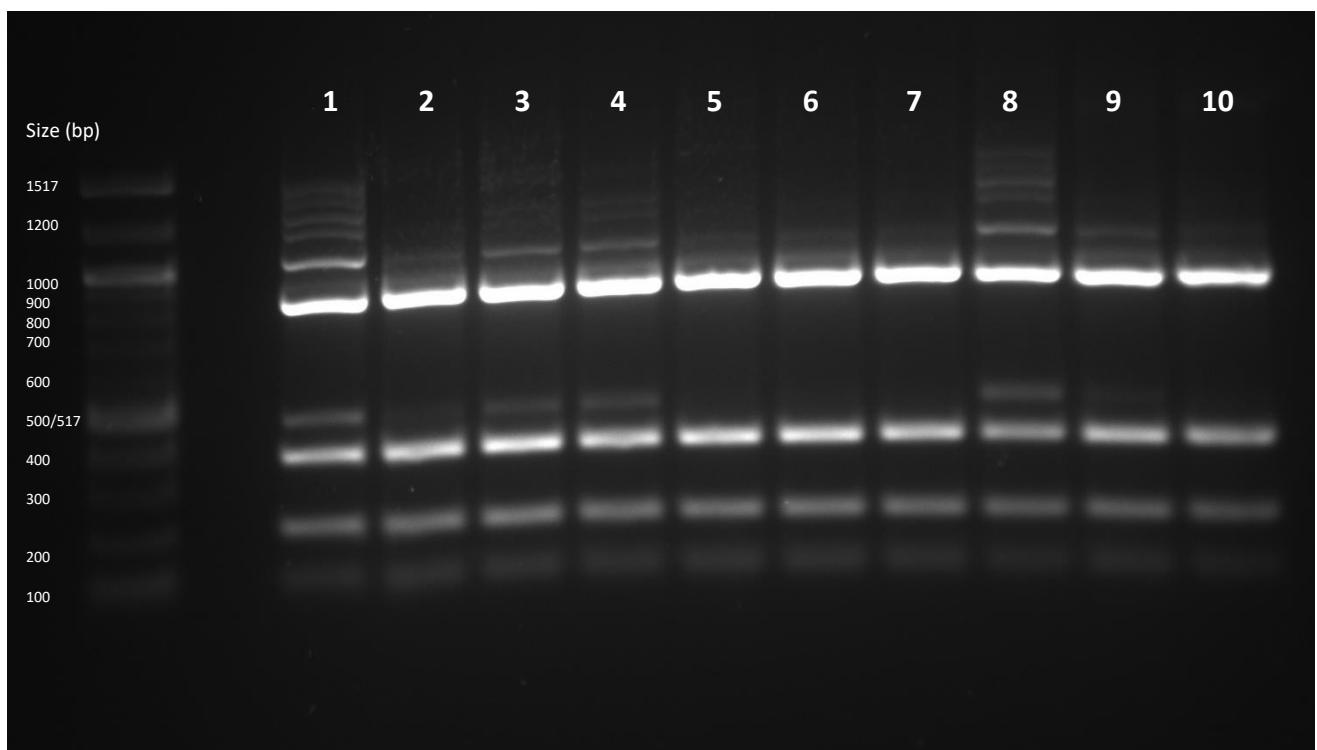
Primer name	Primer Sequence
16S-27F	AGAGTTGATCCTGGCTAG
16S-1492R	TACCTTGTACGACTT
RM2_F1	TCTTCCCACTGCTGACGAAC
RM2_R1	AGTGTGACGGAATCGTTGCT
RM2_F2	CGCCCGAGATACAACATCCA
RM2_R2	GTTGCTTTCTCGACGTGCT
CMCap_F	GGCACTGTTGCAAATAGTCGGT
CMCap_R	GGCACTGTTGCAAAGTTAGCGA
CMCapRE_F	AAAAAGAAATTGGCACTGTTGCAAATAGTCGGT
CMCapRE_R	AAAAAGGTACCGGCAGTGGCAAAGTTAGCGA
CapCon_F1	GGGGAAACGCCCTGGTATCTT
CapCon_R1	TGACGGAATCGTTGCTGTTG
CapCon_F2	GCTGGGAATAGAACAGCCGA
CapCon_R2	CGGCTCGTATGTTGTGGGA
uidA_F	TCTGGCAACCAGGGTGAAG
uidA_R	TAGATATCACACTCTGTCTGGCT
TEM_F	GGAACCGGAGCTGAATGAAG
TEM_R	TCAGCAATAACCAGCCAGC

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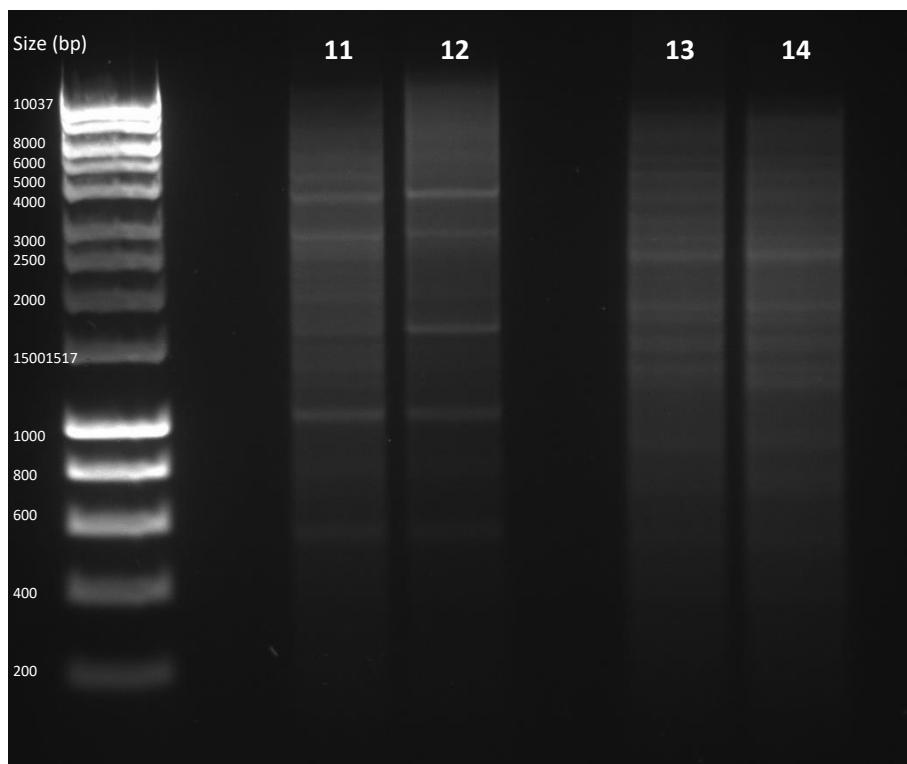
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OXA-1_R	TGCGAAACCAAACAACAGA
tetD_F	AGCAGAAACAAGAAAGCGCA
tetD_R	TTCTCACTCAGCCGTTTGC
aac(3)-lla_F	GCATGCCTCACTTAAAGCGA
aac(3)-lla_R	ATTGATTCAGCAGGCCGAAC
aac(6')-lb-cr_F	CCCAGTCGTACGTTGCTCTT
aac(6')-lb-cr_F	CCTCGGGATCATTGAACAGC

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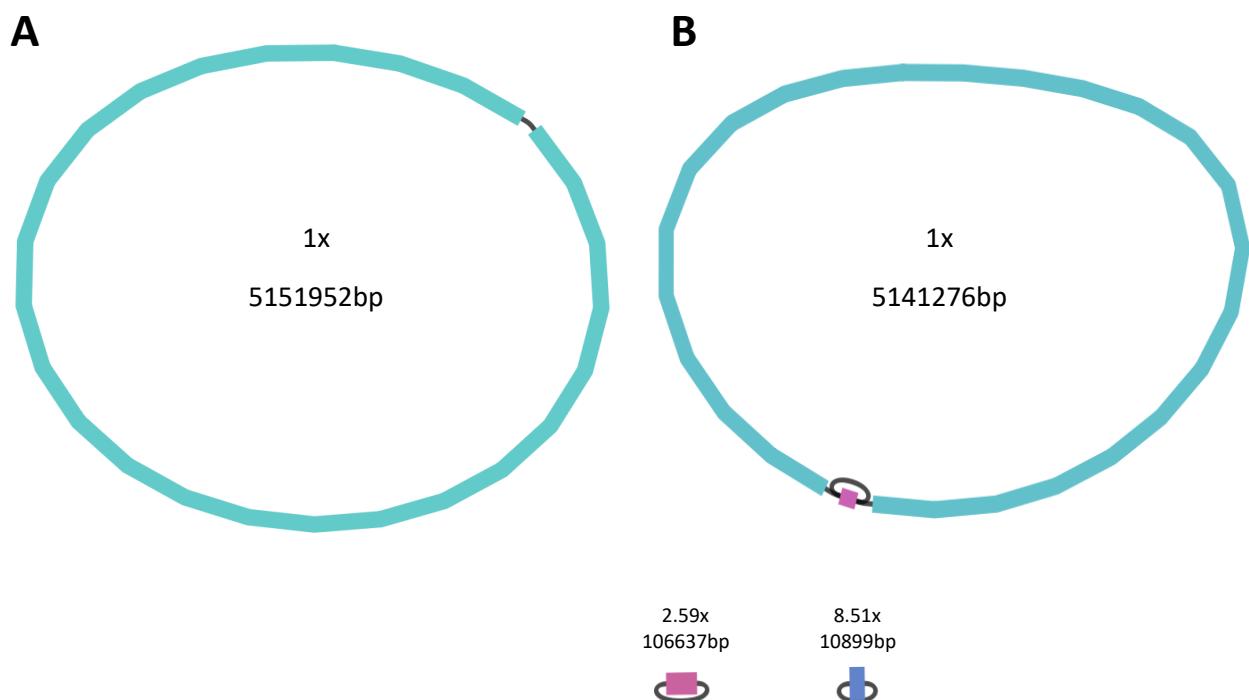
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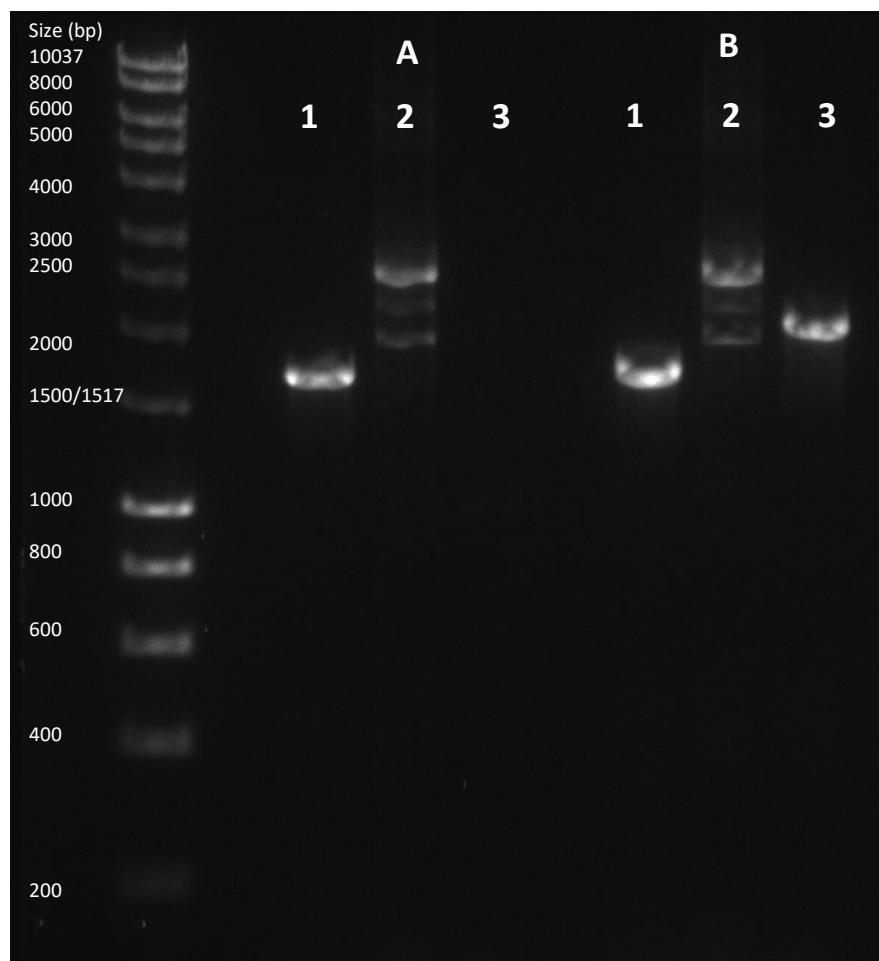
B



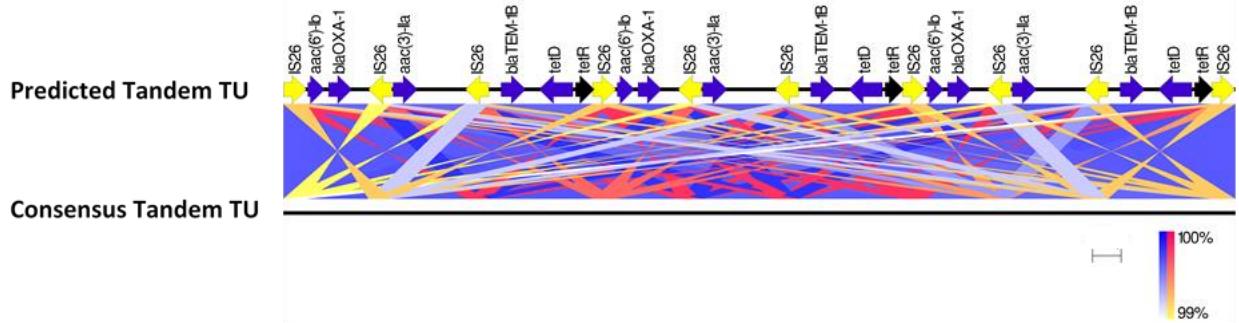
**Supplementary Figure 1: Only one pair isolates are presumptive clones.** Restriction fragment length polymorphism (RFLP) of **A**) 16S rRNA PCR amplicon of **1**) 151985, **2**) 153006, **3**) 101737, **4**) 102167, **5**) 153964, **6**) 152025, **7**) 155512, **8**) 154810, **9**) 190693 and **10**) 169757 digested with AlwNI, PpuHI and MsII. RFLP of **B**) long fragment genomic DNA extraction from **11**) 153964, **12**) 152025, **13**) 190693 and **14**) 169757 digested with Spel and MsII. RFLP was performed as a single replicate ( $n=1$ ). Source data are provided as a Source Data file.



**Supplementary Figure 2: The piperacillin/tazobactam-resistant isolate contained a plasmid and circular DNA molecule which were not present in the piperacillin/tazobactam-susceptible isolate.**  
Visualisation of the hybrid assembly of long and short read sequencing reads by Unicycler of the **A**) piperacillin/tazobactam (TZP)-susceptible isolate and **B**) and TZP-resistant isolate.

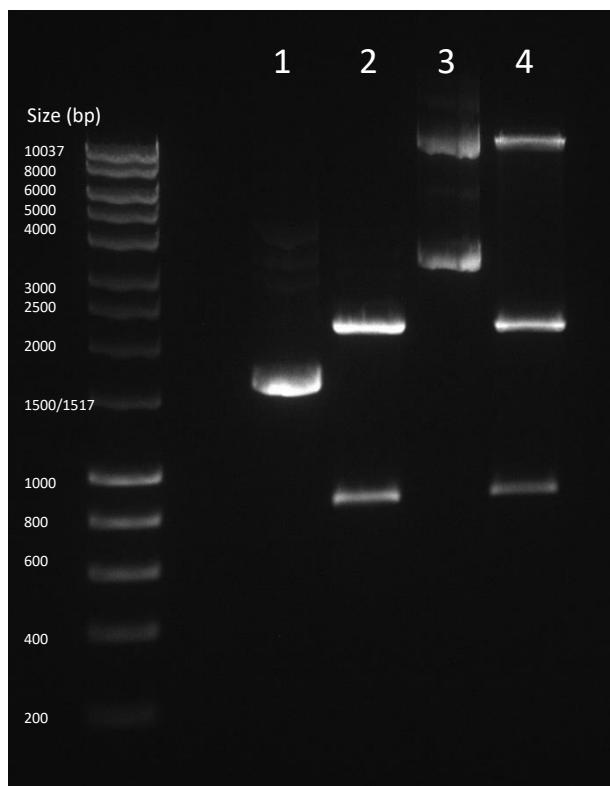


**Supplementary Figure 3: PTn6762 is present in the chromosome of both the piperacillin/tazobactam-resistant and piperacillin/tazobactam-susceptible isolate but exists as a tandem array in the resistant isolate only.** Gel electrophoresis of the PCR amplicons of the **1**) left junction of the chromosomally located IS26 and resistance module, **2**) right junction of the chromosomally located IS26 and resistance module and **3**) translocatable unit (TU) tandem array from **A**) piperacillin/tazobactam (TZP)-susceptible isolate and **B**) TZP-resistant isolate. PCR amplification was performed as a single replicate ( $n=1$ ). Source data are provided as a Source Data file.

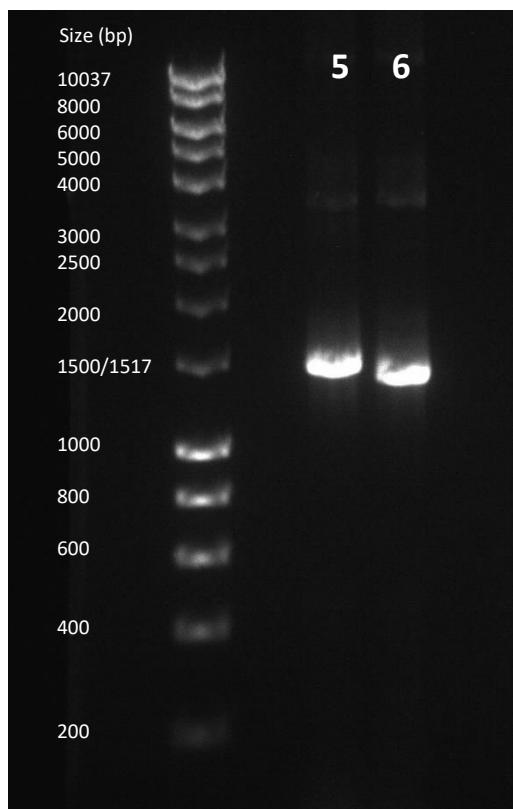


**Supplementary Figure 4: The translocate unit exists as a tandem array in the chromosome of the piperacillin/tazobactam resistant isolate.** Comparison of the nucleotide sequence of the predicted structure of three tandem translocatable units (TU) to the consensus sequence built from mapped Oxford Nanopore Technologies sequencing reads. Yellow arrows represent IS26, blue arrows represent antimicrobial resistance genes and black arrows represent *tetR*. Percentage relatedness between the predicted and consensus sequences in the same orientation are represented in by the blue to white scale and percentage relatedness between the predicted and consensus sequences in the opposite direction are represented in the by the pink to yellow scale. Scale equals 1000 base pairs.

A)



B)



**Supplementary Figure 5: The translocatable unit was captured in the pHSG396:IS26 plasmid following replication of the evolutionary event.** **A)** Gel electrophoresis of **1**) undigested pHSG396:IS26, **2**) pHSG396:IS26 digested with Xhol and EcoRI, **3**) undigested pHSG396:IS26 after growth in the presence of 8/4 µg/ml piperacillin/tazobactam (TZP) and transformation in New England Biolabs (NEB)<sup>®</sup> 5-alpha competent *Escherichia coli* and **4**) pHSG396:IS26 digested with Xhol and EcoRI after growth in the presence of 8/4 µg/ml TZP and transformation in NEB<sup>®</sup> 5-alpha competent *E. coli* and **B)** gel electrophoresis of PCR amplicons from the **5**) left and **6**) right junctions of pHSG396:IS26 following insertion of the translocatable unit. Restriction digest and PCR amplification were performed as a single replicate (*n*=1). Source data are provided as a Source Data file.

A

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B

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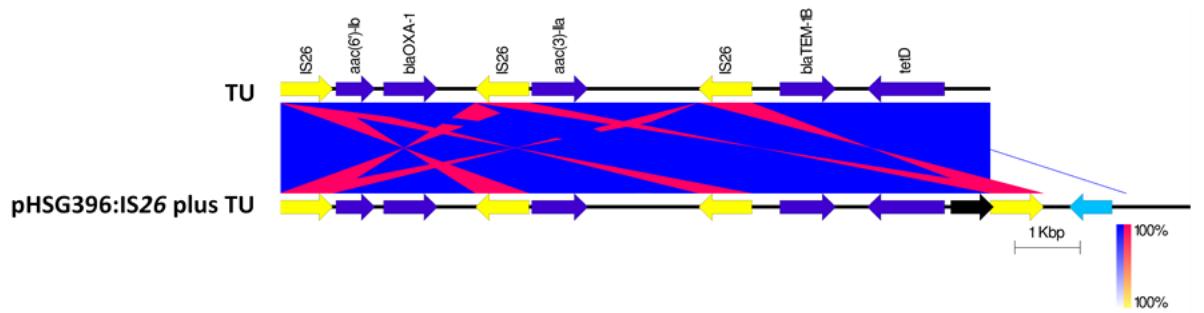
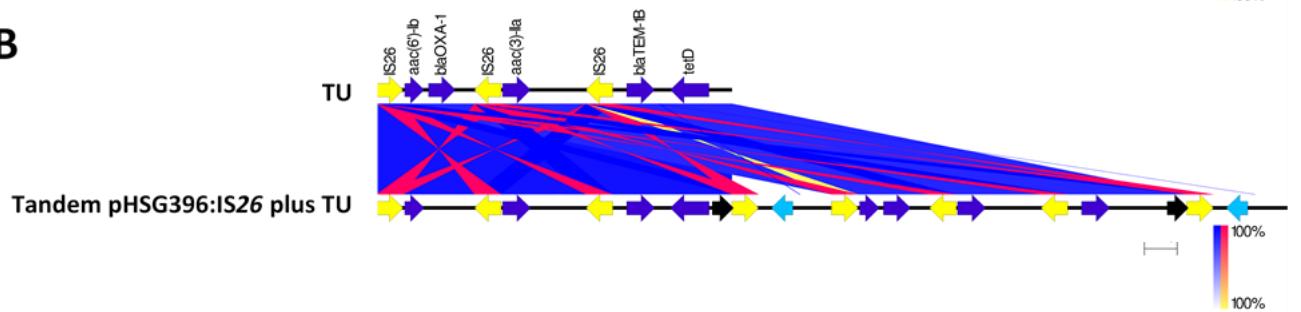
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GAGCGCAGATAACCAAAACTGTTCTTAGTGTAGCCGTAGTTAGGCCACCACTCAAGAACTCTGTAGCACC  
GCCTACATACCTCGCTCTGCTAACCTGTTACAGTGGCTGCTGCCAGTGGCGATAAGTCGTCTTACCGGGT  
TGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGTCGGCTGAACGGGGGTTCGTGCACACAGCCC  
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GCCTTGCTCACATGTTCTTCTGCGTTATCCCTGATTCTGTGGATAACCGTATTACGCCCTTGAGTGAGC  
TGATACCGCTCGCCGAGCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAAGCTCATT  
CGCCATTCAAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGCCCTTCGCTATTACGCCAGCTGGCGA  
AAGGGGGATGTGCTGCAAGCGATTAAGTGGTAACGCCAGGGTTCCAGTCACGACGTTGAAAACGA  
CGGCCAGTGAATT

**Supplementary Figure 6: Sequence of pHSG396:IS26 with the translocatable unit (TU) and tandem array of TUs in the chromosome.** Consensus sequence of **A**) pHSG396:IS26 plasmid, **B**) pHSG396:IS26 plus translocatable unit (TU) and **C**) tandem pHSG396:IS26 plus TU

**A****B**

**Supplementary Figure 7: The translocatable unit inserted adjacent to IS26 in pHSG396:IS26 following replication of the evolutionary event.** Comparison of the nucleotide sequence of the translocatable unit (TU) to the consensus sequence of the predicted plasmid structures of **A)** pHSG396:IS26 plus TU and **B)** tandem pHSG396:IS26 plus TU. Yellow arrows represent IS26, blue arrows represent antimicrobial resistance genes, black arrows represent *tetR* and light blue arrows represents the chloramphenicol resistance gene of pHSG396. Percentage relatedness between the predicted and consensus sequences in the same orientation are represented in by the blue to white scale and percentage relatedness between the predicted and consensus sequences in the opposite direction are represented in the by the pink to yellow scale. Scale equals 1000 base pairs.